#7

Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara

JÖRG SCHNEIDER[1], SARAH C. GILBERT[2], TOM J. BLANCHARD[3], TOMAS HANKE[1], KATHRYN J. ROBSON[4], CAROLYN M. HANNAN[1], MARION BECKER[3], ROBERT SINDEN[5], GEOFFREY L. SMITH[3] & ADRIAN V.S. HILL[1,2]

[1]*Molecular Immunology Group, Institute of Molecular Medicine, Nuffield Department of Medicine, University of Oxford. John Radcliffe Hospital, Oxford OX3 9DS, UK.*
[2]*Wellcome Trust Centre for Human Genetics, University of Oxford, Windmill Road, Headington, Oxford OX3 7BN, UK.*
[3]*Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK.*
[4]*MRC Molecular Haematology Unit, Institute of Molecular Medicine, University of Oxford, Oxford OX3 9DS, UK.*
[5]*Molecular and Cellular Parasitology Group, Department of Pure and Applied Biology, Imperial College, London, UK.*
Correspondence should be addressed to J.S.; e-mail: joerg.schneider@ndm.ox.ac.uk

Immunization with irradiated sporozoites can protect against malaria infection and intensive efforts are aimed at reproducing this effect with subunit vaccines. A particular sequence of subunit immunization with pre-erythrocytic antigens of *Plasmodium berghei*, consisting of single dose priming with plasmid DNA followed by a single boost with a recombinant modified vaccinia virus Ankara (MVA) expressing the same antigen, induced unprecedented complete protection against *P. berghei* sporozoite challenge in two strains of mice. Protection was associated with very high levels of splenic peptide-specific interferon-γ-secreting CD8+ T cells and was abrogated when the order of immunization was reversed. DNA priming followed by MVA boosting may provide a general immunization regime for induction of high levels of CD8+ T cells.

The observation that mice and humans can be protected from malaria by immunization with irradiated sporozoites[1] has led to attempts to develop protective subunit vaccines against the pre-erythrocytic antigens of *Plasmodia*. Indirect evidence from genetic and immunological studies in humans[2,3] and more direct studies in murine malaria models[4–6] have implicated MHC class I restricted CD8+ T cells as major effectors mediating a protective immune response. For the most widely used models of rodent malaria, *P. berghei* and *P. yoelii* in BALB/c mice, many antigen delivery systems designed to induce a protective CD8+ T cell response against liver stages of malaria have been evaluated with variable success.

Intramuscular injection (i.m.) of plasmid DNA has provided encouraging results. Repeated i.m. injection of plasmid DNA encoding pre-erythrocytic antigens conferred medium to high levels of protection in some mouse strains against *P. yoelii* sporozoite challenge[7–9]. However, complete protection was not observed and in some strains protection was poor, particularly in C57BL/6 mice in which no protection has been reported against *P. berghei* or *P. yoelii* using plasmid DNA or live vector immunization.

Recombinant vaccinia viruses expressing antigens derived from pathogens have often been used to induce cellular immune responses that may be protective[10–13]. However in both *P. berghei* and *P. yoelii* models, immunization with recombinant vaccinia virus of the virulent laboratory Western Reserve (WR) strain failed to induce a protective immune response[14,15]. The modified virus Ankara (MVA) strain of vaccinia was attenuated by multiple serial passages, and has lost 31 kb of DNA including host range genes and genes encoding cytokine receptors[16–21]. MVA is replication-restricted and appears to be particularly safe based on studies in some 120,000 humans during smallpox immunization program[17].

As part of a larger study of the immunogenicity and protective efficacy of numerous antigen delivery systems. we have investigated prime-boost immunization strategies with combinations of various recombinant vaccinia virus strains and plasmid DNA. Using plasmid DNA priming and recombinant MVA boosting, complete protection against sporozoite challenge was observed in both BALB/c and C57BL/6 mice. This specific order of immunization was essential for protection. Protection was observed with both the *P. berghei* antigens thrombospondin-related adhesive protein (PbTRAP) and the circumsporozoite protein[22,23] (PbCS) as well as with recombinants sharing only a single nonamer CTL epitope.

Protection after DNA priming and MVA boosting

In an initial screening experiment, all combinations of DNA and MVA vectors expressing PbCSP and PbTRAP were tested for their protective capacity. Groups of mice were immunized with plasmid DNAs (i.m.) or recombinant MVA (i.v.) expressing the two pre-erythrocytic antigens PbCSP and PbTRAP. Eighteen days later the mice were boosted and four weeks after the boost the animals were challenged with 2000 *P. berghei* sporozoites. The homologous combination f DNA followed by DNA did n t protect. In the group of mice immunized with MVA followed by MVA four out of five mice developed blood stage parasitemia. None of the mice that received MVA followed by DNA were protected, but the combination of DNA followed

by MVA protected all animals from challenge with *P. berghei* sporozoites ($P = 0.008$) (Table 1*a*). All mice immunized with DNA and MVA expressing the control protein β-galactosidase in an identical sequence developed blood stage parasitemia after nine days.

To identify which of the pre-erythrocytic antigens confer protection, mice were again immunized with DNA followed by a boost with MVA, both encoding the same single antigen. All ten mice immunized with a mixture of plasmid DNAs expressing PbCSP and PbTRAP, and boosted with recombinant MVA expressing PbCSP and PbTRAP, were completely protected (Table 1*b*) as before. Similarly the group that received the combination of DNA followed by MVA expressing PbCSP was completely protected. In mice immunized with PbTRAP, 14 out of 16 mice were protected (Table 1*b*) indicating that this antigen, recently identified in *P. berghei*[23], is of protective relevance.

A single CD8+ T cell epitope confers protection

The mice immunized with DNA and MVA expressing complete *P. berghei* antigens developed antibodies against *P.berghei* sporozoites (data not shown) as well as T cell responses. To determine whether the protection from sporozoite challenge could be mediated entirely by MHC class I-restricted T cells, a group of mice was immunized with plasmid DNA and recombinant MVA that expressed a single H-2K^d-restricted *P. berghei* epitope SYIPSAEKI as part of a string of different human and murine CTL epitopes deriving from *P. falciparum* and HIV/SIV (see Methods). All 11 mice in this group were protected from sporozoite challenge (Table 1*b*), demonstrating that an immune response to this CTL epitope alone is sufficient for complete protection. Mice immunized with plasmid DNA and recombinant MVA expressing *E. coli* β-galactosidase were not protected.

Protection depends upon the vaccinia virus strain

To determine whether the strain of vaccinia employed was of relevance in inducing protection, mice were immunized with plasmid DNA expressing PbCSP followed by a boost with the different PbCSP expressing recombinant vaccinia strains, MVA, the virulent WR strain and the attenuated NYVAC strain. In a preliminary experiment no significant protection was observed on i.v. boosting with 10^4 pfu of the replication-competent WR strain of vaccinia (1/10 mice protected) whereas significant protection was observed on boosting with either recombinant MVA (10^6 pfu, i.v.) or NYVAC (10^6 pfu, i.v.) (80% and 60% respectively), both replication-defective strains. Because NYVAC and MVA are vaccine candidates for humans, the latter two groups were then compared in a larger study. Significantly better protection ($P = 0.01$) was observed by boosting with recombinant MVA than with the NYVAC recombinant (Table 2).

The protection is maintained on re-challenge

Groups of mice immunized with DNA followed by MVA that survived sporozoite challenge were re-challenged after 28–35 days to determine whether protection was maintained. The great majority of the animals were protected from sporozoite re-challenge (Table 3). Protection was again observed in mice immunized by both antigen-based constructs and constructs that express, from *P. berghei*, only the CD8+ T cell epitope SYIPSAEKI (Table 3*b*).

The route of MVA boosting influences protection

To assess whether protection could be induced by recombinant MVA delivered by other routes, BALB/c mice were primed with plasmid DNA encoding a single antigen, PbCSP and boosted either i.v., intradermally (i.d.) or i.m. with MVA-PbCSP, and then challenged with infectious sporozoites. Mice boosted with recombinant MVA-PbCSP i.v. were completely protected as before. 80% of the mice boosted i.d. and 50% of the mice boosted i.m. were protected using this low dose (10^6 pfu) boost (Table 4). Mice immunized with DNA followed by MVA expressing the influenza nucleoprotein and β-galactosidase were not protected.

Table1 Protection of BALB/c mice after immunization with plasmid DNA followed by recombinant MVA

	Immunization 1	Immunization 2	No. animals inf./ no. challenged	% protection
a				
Antigens used: PbCSP + PbTRAP				
	DNA	DNA	5/5	0
	MVA	MVA	4/5	20
	DNA	MVA	0/5	100
	MVA	DNA	5/5	0
Control mice immunized with β-galactosidase				
	DNA	DNA	5/5	0
	MVA	MVA	5/5	0
	DNA	MVA	5/5	0
	MVA	DNA	5/5	0
b	DNA	MVA		
	CSP + TRAP	CSP + TRAP	0/10	100
	CSP	CSP	0/10	100
	TRAP	TRAP	2/16	88
	epitope	epitope	0/11	100
	β-galactosidase	β-galactosidase	6/7	14
	none	none	9/10	10

Results of two challenge experiments (*a* and *b*) using different immunization regimes of plasmid DNA and MVA as indicated. BALB/c mice were used in all cases. The immunization doses were 50 μg of plasmid DNA and 10^7 pfu (*a*) or 10^6 pfu (*b*) of recombinant MVA expressing the antigens indicated. The interval between immunizations 1 and 2 was 21 days (*a*) or 14 days (*b*). Challenges were performed at 29 (*a*) or 18 (*b*) days after the last immunization by i.v. injection of 2000 *P.berghei* sporozoites and blood films assessed at five, eight and ten days post challenge. CSP and TRAP indicate the entire *P. berghei* antigens and "epitope" indicates the polyepitope constructs containing the *P.berghei* CTL epitope described in methods.

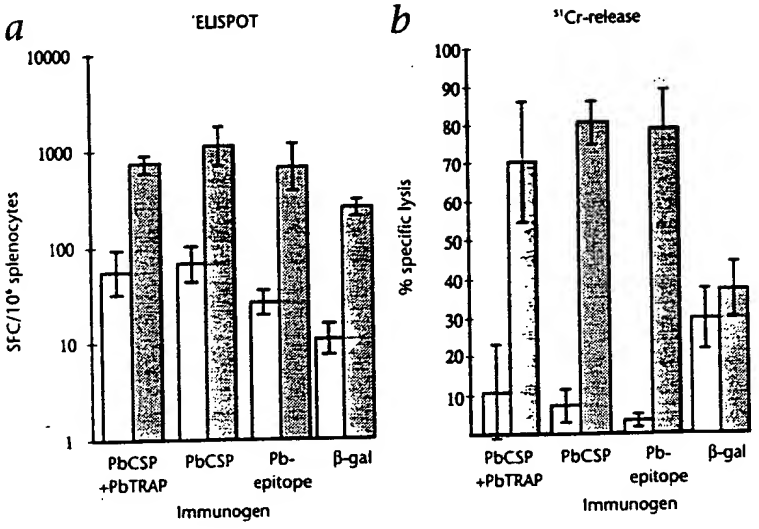
Highly susceptible C57BL/6 mice are protected

C57BL/6 mice are very susceptible to *P. berghei* sporozoite challenge[24]. C57BL/6 mice were immunized using the DNA-MVA prime boost regime with both pre-erythrocytic antigens PbCSP and PbTRAP, and challenged with either 200 or 1000 infectious sporozoites per mouse. (Two hundred sporozoites corresponds to more than twice the dose required to induce infection in these mice[24]). All ten mice challenged with 200 sporozoites showed sterile immunity. Even in the group challenged with 1000 sporozoites, 60% of the mice were protected (Table 5). All the naive C57BL/6 mice were infected after challenge.

Table 2 Influence of different recombinant vaccinia strains on protection.

Immunization 1	Immunization 2	No. animals inf./ no. challenged	% protection
DNA			
CSP	MVA (CSP)	*5/40	88
CSP	NYVAC (CSP)	*15/40	63
β-galactosidase	MVA (NP)	8/8	0

*Cumulative data from two independent experiments
Challenge of DNA-primed BALB/c mice boosted with two different replication-defective vaccinia virus strains expressing PbCSP. The immunization doses were 50 μg of plasmid DNA and 10^6 pfu of NYVAC and MVA. The interval between immunizations 1 and 2 was 23 days. Challenges were performed at 28 days after the last immunization by i.v. injection of 2000 *P. berghei* sporozoites and blood films assessed at 7, 9 and 11 days post challenge. CSP indicates the entire *P. berghei* antigen and NP the nucleoprotein of influenza virus.



...g of peptide specific CD8+ T cell responses in ...DNA priming (open bars) and boosting with ... (shaded bars). Animals were immunized with ...ing the antigen(s) indicated on the X-axis. ...ope string including the CTL epitope from *P.* ...CD8+ T cell responses were measured using the ...say on fresh splenocytes incubated for 22 hr ...icted peptide SYIPSAEKI from *P. berghei* CSP ...PbTRAP, PbCSP and epitope) and the L^d re- ...TPHPAPRIGL from *E. coli* β-galactosidase (group ...are expressed as number of spot forming cells ...sent data derived from three mice assayed indi- ...enocytes from the same mice were also assayed in ...Cr-release assays at an effector to target ratio of ...days of *in vitro* restimulation with the same pep- ...). The mice monitored for CD8+ T cell responses ...the same groups that were challenged at the same ...in Table 1*b*.

...ll induction

...ell responses induced by the various immunization regimes ...nitored using ELISPOT and ^{51}Cr-release assays. The ELISPOT ...used to detect peptide-specific, interferon γ- (INF-γ) secret- ...without *in vitro* restimulation. The protective relevance of ...ly sensitive assay is supported by the observation that INF- ...ibit parasite development *in vivo*[25], and the recent finding ...ne knock-out studies, elimination of *P. berghei* liver stages ...pendent of Fas-mediated or perforin-mediated cytotoxic- ...wever, antigen specific CD8+ cytotoxic T lymphocytes (CTL) ...n reported to recognise and lyse infected murine hepato- ...*vitro*[27]. So, splenocytes were also restimulated for seven days ...ptide SYIPSAEKI *in vitro* and their cytolytic activity was ...in ^{51}Cr-release assays. Three mice from each of the groups ...hallenge experiment of Table 1*b* were studied both after DNA ... (Fig. 1 *a* and *b*, white bars) and following the MVA boost ...and *b*, black bars). No PbTRAP-derived CD8+ T cell epitope ...defined to date but CD8+ T cells specific for the PbCSP ...lactosidase epitopes could be clearly detected in ELISPOT ...their frequency was below 100 spot forming cells (SFC) per ...nocytes (Fig. 1*a*). Splenocytes from the same mice were also ...lated with peptide *in vitro*. As expected from the relatively ...ecursor frequency detected with the ELISPOT assay, not all the ...cyte cultures showed significant cytotoxicity in conventional ...ease assays (Fig. 1*b*). However, the CD8+ T cell levels 18 days ...e MVA boost were remarkably high. The frequencies of SYIP- ...pecific CD8+ T cells ranged between 300–1000 SPC/10^6 ...ytes (Fig. 1*a*). These frequencies resulted in high level of spe- ...ls in ^{51}Cr-release assays (Fig. 1*b*). A similar, but less pronounced ...as observed for the β-galactosidase epitope.

...etermine the influence of the immunization regime on the ...cell frequency, mice were immunized with plasmid DNA ...combinant MVA in different combinations. The highest level ...tide specific CD8+ T cells 885 +/- 85 (mean +/- SEM) was ...ed by priming with plasmid DNA and boosting with the recom- ...nt MVA. This was markedly more immunogenic than the reverse ...of immunization (MVA/DNA: 100 +/- 23; $P = 8.6 \times 10^{-10}$) or ...ng with the homologous antigen delivery system (MVA/MVA: ...50; DNA/DNA: 30 +/- 9; $P = 1.4 \times 10^{-8}$ and 2.96×10^{-10} respec- ...). It was also more immunogenic than the DNA and MVA ...nizations given simultaneously (Fig. 2). In a second experi- ...sing ten mice per group the enhanced immunogenicity of ...NA/MVA sequence was confirmed: DNA/MVA 856 +/- 201; MVA/DNA 168 +/- 72; MVA/MVA 345 +/- 90; DNA/DNA 92 +/- 46.

To assess the influence of the route of MVA administration on CD8+ T cell immunogenicity, mice were primed i.m. with DNA as before and boosted by five different routes with recombinant MVA. Significantly higher immunogenicity was observed for the intravenous and intradermal routes than for intramuscular, intraperitoneal or subcutaneous routes (Fig. 3).

Discussion

In addition to their role in antiviral immunity there is increasing evidence that CD8+ T cells play an important part in the immune response to many intracellular bacterial and protozoan pathogens and to some tumors. Traditionally, vaccine formulations were designed to primarily induce antibodies but recently efforts have been aimed at inducing strong cellular immune responses. This has proved particularly difficult for CD8+ T cell responses.

Live replicating viral vectors have been used to induce CD8+ T cell responses but most of these recombinants, such as influenza and unattenuated vaccinia, are unlikely to have a safety profile adequate for widespread use as prophylactic vaccines in humans. The discovery of the immunogenicity of plasmid DNA for CD8+ T cell induction offers a promising new approach. However, immunogenicity in rodent models of malaria has been relatively limited; several immunizations have usually been required and complete protection has not been observed in any mouse strain[9]. More limited data from DNA vaccine studies in primates suggest that immunogenicity for CD8 T cell responses is weaker in macaques and chimpanzees than in mice

Table 3 Re-challenge of mice that survived first challenge

	Immunization 1 DNA	Immunization 2 MVA	No. animals inf./ no. challenged	% protection
a				
	CSP + TRAP	CSP + TRAP	0/5	100
	none	none	4/5	20
b				
	CSP + TRAP	CSP + TRAP	1/8	87
	CSP	CSP	0/10	100
	epitope	epitope	2/11	82
	none	none	4/5	20

Surviving BALB/c mice were re-challenged 35 days (*a*) or 28 days (*b*) after the first challenge with 2000 *P. berghei* sporozoites. Blood films were assessed at 7, 9 and 11 days post challenge. Mice in *a* and *b* were surviving mice from Table 1*a* and 1*b* respectively. The group that received PbTRAP was not re-challenged. For immunization regimens and intervals see Table 1.

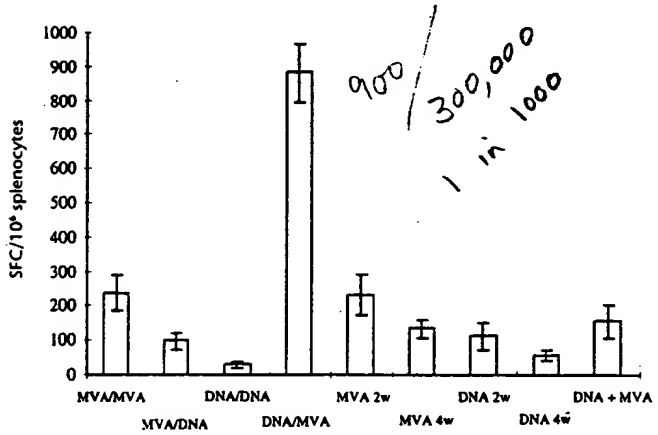


Fig. 2 Frequencies of peptide-specific CD8+ T cells following different immunization regimes. Results are shown as the number of spot forming cells (SFC) per one million splenocytes. Mice were immunized with either plasmid DNA expressing PbCSP or PbCSP expressing MVA or combinations of the two as shown on the X axis, at two week intervals and the number of splenocytes specific for the SYIPSAEKI peptide determined in INF-γ ELISPOT assays two weeks after the last immunization. Each bar represents the mean number of SPCs from three mice assayed individually.

(J.S. & T.H., unpublished data).

We report here that much higher levels of CD8+ T cells are induced by boosting a DNA-primed immune response with MVA than with the same plasmid DNA and that this enhanced immunogenicity is associated with a striking difference in protective efficacy against malaria sporozoite challenge. The complete protection observed in the highly susceptible C57BL/6 mice is particularly encouraging as no vaccination protocol designed to induce strong cellular immune responses has previously protected this strain of mice against either *P. berghei* or *P. yoelii*. Two immunizations with MVA were not significantly more immunogenic than a single immunization, but were better than two DNA immunizations. The lack of boosting after repeated DNA immunizations has been reported previously[6] and is not understood. The lack of boosting with repeated MVA immunizations may relate to antibodies induced against the virus.

The mechanism of the enhanced protective efficacy of DNA priming and MVA boosting compared to the reverse sequence is unclear. It has been reported that recombinant influenza priming and boosting with a recombinant wild type vaccinia induced partial protection against *P. yoelli* infection, but the reverse order of immunization did not[28]. Li *et al.* speculated that the effect might relate to increased homing of CD8+ T cells to the liver following the vaccinia than after the influenza immunization. We observe a marked difference in specific CD8+ T cells in the spleens according to the immunization sequence and it appears unnecessary to invoke homing effects to account for the differential protection. We speculate that the mechanism may be related to the phenomenon of immunodominance as follows: The MVA vector appears more immunogenic than DNA for CD8+ T cell induction (Fig. 2); however, because of the large number of viral genes that are co-expressed, the immune response to the malaria antigen or epitope may not be immunodominant, but priming with just the malaria antigen using plasmid DNA may produce sufficient primed CD8+ T cells to the malaria epitope to allow the recombinant MVA-induced response to focus on this rather than viral epitopes. In keeping with this speculation we find that priming of an immune response with a recombinant Ty virus-like particle and boosting with recombinant MVA is also more immunogenic and protective than the reverse order of immunization (Plebanski *et al.* unpublished data).

Comparing the protective efficacy of boosting a DNA-primed CD8+ T cell response with different strains of recombinant vaccinia viruses expressing the same antigen, revealed a significant difference in protective efficacy between the vaccinia virus strains. Previous studies of recombinants encoding SIV and influenza antigens suggest that MVA may be somewhat more immunogenic and protective than WR-strain vaccinia[11,19,29]. Here, recombinant MVA, and to a significantly lesser extent, recombinant NYVAC[30], were able to boost a DNA-primed CD8+ T cell response to reach protective levels. Intraperitoneal immunization with the NYVAC recombinant alone has been shown to be significantly protective[30]. The attenuation of NYVAC and MVA is a result of large deletions in the viral genome induced by molecular techniques or through serial passage, respectively. MVA does not express an INF-γ receptor (Blanchard *et al.*, manuscript submitted) but this is unlikely to contribute to the enhanced immunogenicity observed here because the INF-γ receptor expressed by strain WR inhibits human but not murine INF-γ[31]. However, MVA also does not express receptors for INF-α/β and TNF and these may influence the development of a cellular immune response within the micro-environment where a CD8+ T cell response is initiated. By secreting cytokine receptors at the site of vaccinia infection, cytokines essential to drive a Th1 response may be locally depleted. MVA also encodes a functional IL-1β receptor and this may interfere with priming of an immune response[32]. MVA elicits local production of type I interferon in primary fibroblasts (T.J.B., A. Alcami, P. Andrea, and G.L.S. unpublished data) and this would be expected to expand CD8+ T cell clones *in vivo* and might contribute to the potent boosting but poorer priming seen with recombinant MVA[33]. As both plasmid DNA and NYVAC virus encoding *P. falciparum* pre-erythrocytic antigens are now at the stage of early clinical trials, our data suggest that prime-boost regimens with these constructs, or preferably with MVA, merit clinical evaluation.

The protective effect of the newly-described PbTRAP antigen further supports the potential of its *P. falciparum* homologue, PfTRAP, as a malaria vaccine candidate. The *P. yoelii* homologue of PbTRAP, PySSP2, was protective (57%) in BALB/c mice when administered in transfected tumor cells[34] but did not protect BALB/c mice from *P. yoelii* sporozoite challenge when given as plasmid DNA[9]. The complete protective efficacy of a single CTL epitope in protecting against sporozoite challenge also supports current attempts to develop polyepitope-based vaccines against malaria. The protective immunity induced here may be long-lived; the great majority of mice that were protected in the first challenge were also protected in a second challenge 28–35 days later. However, further data on the duration of protection and the role of sporozoite exposure will be important.

The marked boosting effect of a single immunization with recombinant MVA may be of particular relevance to immunization against

Table 4 Influence of the route of MVA administration on protective efficacy

Immunization 1 DNA	Immunization 2 MVA	No. animals inf./ no. challenged	% protection
CSP	CSP i.v.	*0/20	100
CSP	CSP i.d	2/10	80
CSP	CSP i.m.	5/10	50
epitope	epitope i.v.	1/10	90
NP	NP i.v.	10/10	0

*Cumulative data from two independent experiments.
All the immunizations were done in BALB/c mice as described previously except for the route of boosting with MVA.PbCSP as indicated.

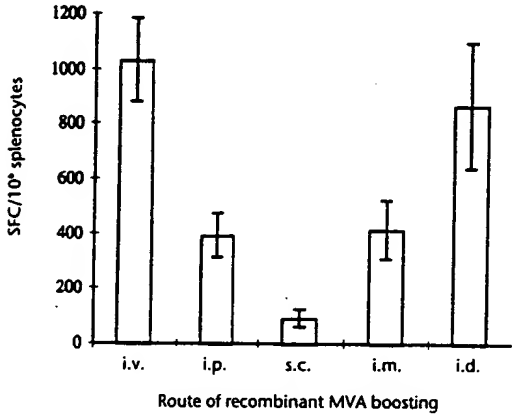


Fig. 3 Frequencies of peptide-specific CD8+ T cells following different routes of MVA boosting. Results are shown as the number of spot-forming cells (SFC) per one million splenocytes. Mice were primed with plasmid DNA (pTH.PbCSP, 50 µg/mouse i.m.) and two weeks later boosted with 10^6 pfu MVA.PbCSP via the indicated routes. The number of splenocytes specific for the SYIPSAEKI peptide was determined in INF-γ ELISPOT assays two weeks after the last immunization. Each bar represents the mean number of SPCs from three mice assayed individually.

malaria in endemic areas. Humans exposed to *P. falciparum* in areas of high transmission develop low to moderate levels of CTL through natural priming from infective mosquito bites[2,3]. The measured precursor frequencies are of the order of 10–100 peptide-specific cells per 10^6 PBMC[35]. Thus it might be possible to boost these levels substantially by using an MVA recombinant without any priming immunization in such populations.

We have very recently extended this work to show that in mice the DNA-MVA prime boost regime is effective at inducing high levels of CD8+ T cells to three further CTL epitopes from other antigens and pathogens (unpublished data). Furthermore, in chimpanzee studies, priming with DNA and boosting with MVA has yielded very high levels of CD8+ T cells to a *P. falciparum* epitope: by ELISPOT analysis more than 3000 IFN-γ releasing cells per million peripheral blood lymphocyte were measured to a PfTRAP peptide (J. S., A. Thomas *et al.*, manuscript in preparation). Plasmid DNA is now being used in human vaccine trials and MVA appears to be an exceptionally safe live virus vector. Therefore this highly immunogenic prime-boost regime holds promise for the prophylactic or therapeutic immunization of humans against diseases in which CD8+ T cells play a protective role.

Meth ds

Plasmid DNA constructs. The DNA vaccine vector pTH, used throughout the study, was derived from pRc/CMV (Invitrogen) by removing the *Bam*HI fragment that contains the SV40 origin of replication and neomycin resistance marker and replacing the CMV promoter with a longer version of the same promoter containing intron A. The resulting plasmid thus contains the CMV promoter with intron A for expression in eukaryotic cells, followed by a multiple cloning site and the bovine growth hormone poly A sequence. The plasmid is incapable of replication in mammalian cells. The genes encoding the complete sequence of *P. berghei* CSP[22] and TRAP[23] were introduced into the multiple cloning site using standard methods to make pTH.PbCSP and pTH.PbTRAP. Plasmids pTH.M and pTH.HM contain synthetic polyepitope strings consisting of a series of minimal T cell epitopes from *P. falciparum* (M), or *P. falciparum* epitopes fused to a string of HIV/SIV epitopes (HM). The M epitope string also contains the H-2K^d-restricted epitope SYIPSAEKI from *P. berghei* CSP. Each polyepitope string has a PK-Tag (Serotech) antibody epitope fused in frame at the C terminus to allow detection of expression of the string in mammalian cells[36]. The expression of pTH.PbCSP and pTH.PbTRAP upon transfection into COS-1 cells was detected by immunofluorescence using antigen-specific polyclonal murine antiserum for PbTRAP or PbCSP followed by FITC-labelled secondary antibodies (data not shown). Control plasmids were pcDNA3/His/LacZ (Invitrogen) encoding *E. coli* β-galactosidase and pTH.UbNPPk expressing the influenza virus nucleoprotein (T.H., unpublished). Plasmid DNA for injections was purified using anion-exchange chromatography (Qiagen, Hilden, Germany) and diluted in endotoxin-free phosphate buffered saline (PBS) (Sigma).

Table 5 Protection of C57BL/6 mice from sporozoite challenge

	No. animals inf./ no. challenged	% protection
1000 sporozoites		
DNA followed by MVA	4/10	60
naive	5/5	0
200 sporozoites		
DNA followed by MVA	0/10	100
naive	5/5	0

Mice were immunized with DNA followed by recombinant MVA expressing PbCSP and PbTRAP as described previously. Fourteen days later the mice were challenged with 1000 or 200 *P. berghei* sporozoites as indicated.

Generation of recombinant vaccinia viruses. MVA was kindly provided by A. Mayr (Veterinary Faculty, University of Munich, Germany). Recombinant and non-recombinant viruses were routinely propagated and titered in chicken embryo fibroblasts (CEF) grown in minimal essential medium supplemented with 10% fetal calf serum (FCS). Recombinant MVA were made as described[37,38] by cloning DNA sequences encoding PbCSP, PbTRAP, influenza NP and the HM polyepitope string into the vaccinia virus shuttle vectors pSC11 and pMCO3. CEF infected with MVA at a multiplicity of 0.05 pfu per cell were transfected with lipofectin (Gibco) and plasmid as described[37,38]. Expression of PbTRAP, influenza nucleoprotein and the HM polyepitope is driven by the vaccinia P7.5 promoter. Expression of PbCSP is driven by the synthetic strong promoter (SSP)[38]. Recombinants were repeatedly plaque purified and expression of the recombinant gene was confirmed using immunofluorescence. The recombinant NYVAC (vP936) and the recombinant Western Reserve vaccinia expressing PbCSP were described previously[4,30].

Cells and culture medium. P815 cells were cultured in RPMI 1640 (Sigma, St Louis, MO) supplemented with 10% heat inactivated FCS. Splenocytes were restimulated as described below in α MEM medium with 10% FCS, 2 mM glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, 50 µM 2-mercaptoethanol and 10 mM Hepes pH 7.2 (all from Gibco, Grand Island, NY).

Peptides. The H-2K^d-restricted epitope from the CS protein of *P. berghei* (aa 372–380) SYIPSAEKI[5], the H-2K^d-restricted epitope (aa 147–155) TYQRTRALV from the nucleoprotein of the influenza A virus[39] and the H-2L^d-restricted epitope (aa 876–884) TPHPARIGL from *E. coli* β-galactosidase[40] were made using a standard Fmoc/t-butyl solid-phase technique on an automated Zinsser Analytical synthesizer (Zinser Analytic, Maidenhead, UK) or purchased from Research Genetics (Huntsville, AL, USA).

Animals and immunizations. Female BALB/c and C57/BL6 mice 4–6 weeks old were obtained from Harlan Orlac (Shaws Farm, Blackthorn, UK). Plasmid DNA (25–50 µg/muscle) dissolved in endotoxin-free PBS (Sigma) was injected into each musculus tibialis under anaesthesia. In early experiments 50 µl of 10 mM cardiotoxin (Latoxan, France) was injected into the muscle 5–9 days prior to immunization[41] but this was found not to influence either immunogenicity or protective efficacy and was thereafter omitted. Recombinant vaccinia virus was diluted in endotoxin-free PBS/2% BSA and the amounts indicated were injected i.v. into the lateral tail vein, intramuscularly, intradermally, subcutaneously or intraperitoneally.

ELISPOT assays. The number of INF-γ-secreting, peptide-specific T cells in fresh splenocyte preparations was determined by the ELISPOT method[42]. Briefly, 96-well nitrocellulose plates (Milliscreen MAHA, Millipore, Bedford, MA) were coated with 15 µg/ml of the anti-mouse INF-γ mAb R4-6A2 (hybridoma purchased from the European Collection of Animal Cell Cultures), in 50 µl of phosphate-buffered saline (PBS). After overnight incubation at 4 °C, the wells were washed with PBS and blocked for 1 hr at room temperature with 100 µl

RPMI/10% FCS. Splenocytes from immunized mice were resuspended to 1–2 × 10^7 cells/ml and placed in duplicates into the antibody-coated wells and serially diluted. Peptide (final concentration of 1 µg/ml) was added to each test well. Control wells contained an irrelevant peptide. After overnight incubation the plates were washed six times with PBS and once with water, incubated for 3 hr with a solution of 1 µg/ml of biotinylated anti-mouse INF-γ mAB XMG1.2 (Pharmingen, CA), washed, and incubated for 2 hr with 50 µl of a 1 mg/ml solution of Streptavidin-Alkaline-Phosphatase polymer (Sigma) both at R.T. Spots were developed by adding 50 µl of an alkaline phosphatase conjugate substrate solution (Biorad, Hercules, CA), and reactions were stopped by washing with water. Spots were counted using a stereomicroscope.

CTL assays. Splenocytes (5 × 10^6 cells/ml) from immunized mice were incubated in six-well tissue culture plates with 1 µg/ml of the respective peptide. At day 3 of culture 10 U/ml of human IL-2 (Lymphocult-T, Biotest, Dreieich, Germany) was added. At days 5–7 the restimulated splenocytes were used in standard ^{51}Cr-release assays[42] using syngenic peptide loaded P815 cells as target cells.

***P. berghei* challenge.** Sporozoites of *P. berghei* (ANKA strain clone 1) were obtained from laboratory reared female *Anopheles stephensi* mosquitoes maintained at 18 °C for 20–25 days after feeding on infected mice. Salivary glands from the mosquitoes were collected by hand dissection and placed into a tissue homogeniser to release the sporozoites. Sporozoites were released into RPMI and counted using a hematocytometer. Mice were challenged by injection of sporozoites into the lateral tail vein 2–3 weeks after the last immunization. Infection was determined by observing the appearance of ring forms in Giemsa stained blood smears taken at the days indicated post-challenge. If blood stage parasitemia was observed at two time points the mice were killed. Protected mice were observed at later time points for signs of illness and if protection persisted they were re-challenged.

Statistical analysis. In protection studies, the number of mice protected in each group was compared using a χ^2 test in the Statcalc program in the EpiInfo Version 5 package. In immunogenicity studies the ELISPOT responses were compared between groups using Student's t test with the Microsoft Exel Version 5.0a package.

Acknowledgments

We thank Jiang-Ting Hu, G. Butcher and C. Parker for assistance, E. Paoletti (Virogenetics, New York) for providing the NYVAC recombinant, V. Satchidanandam and B. Moss (NIH, Bethesda) for providing the WR-CSP vaccinia recombinant, Magda Plebanski and Andrew McMichael (Oxford) for discussions and the Wellcome Trust, the UK Medical Research Council and the European Commission (IC18-CT95-0019 TMR fellowship for J.S.) for support. AVSH is a Wellcome Trust Principal Research Fellow.

RECEIVED 5 AUGUST 1997; ACCEPTED 4 FEBRUARY 1998

1. Nussenzweig, V. & Nussenzweig, R.S. Rationale for the development of an engineered sporozoite malaria vaccine. *Adv. Immunol.* 45, 283–334 (1989).
2. Hill, A.V.S. *et al.* Molecular analysis of the association of HLA-B53 and resistance to severe malaria. *Nature* 360, 434–439 (1992).
3. Aidoo, M. *et al.* Identification of conserved antigenic components for a cytotoxic T lymphocyte-inducing vaccine against malaria. *Lancet* 345, 1003–1007 (1995).
4. Rodrigues, M.M. CD8+ cytolytic T cell clones derived against the Plasmodium yoelii circumsporozoite protein protect against malaria. *Int. Immunol.* 3, 579–585 (1991).
5. Romero, P. *et al.* Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein and protect against malaria. *Nature* 341, 323–326 (1989).
6. White, K.L., Snyder H.L. & Krzych U. MHC class I-dependent presentation of exoerythrocytic antigens to CD8+ T lymphocytes is required for protective immunity against Plasmodium berghei. *J. Immunol.* 156, 3374–3381 (1996).
7. Doolan, D.L. *et al.* Circumventing genetic restriction of protection against malaria with multigene DNA immunization: CD8+ cell-, interferon gamma-, and nitric oxide-dependent immunity. *J. Exp. Med.* 183, 1739–1746 (1996).
8. Sedegah, M., Hedstrom, R., Hobart, P. & Hoffman, S. L. Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein. *Proc. Natl. Acad. Sci. USA* 91, 9866–9870 (1994).
9. Doolan, D.L. & Hoffmann, S.L. Multi-gene vaccination against malaria: A multistage, multi-immune response approach. *Parasitol. Today* 13, 171–178 (1997).
10. Bennink, J.R. *et al.* Recombinant vaccinia virus primes and stimulates influenza haemagglutinin-specific cytotoxic T cells. *Nature* 311, 578–579 (1984).
11. Bender, B.S. *et al.* Oral immunization with a replication-deficient recombinant vaccinia virus protects mice against influenza. *J. Virol.* 70, 6418–24 (1996).
12. Thomson, S.A. *et al.* Recombinant polyepitope vaccines for the delivery of multiple CD8 cytotoxic T cell epitopes. *J. Immunol.* 157, 822–826 (1996).
13. An, L.L., Pamer, E. & Whitton, J.L. A recombinant minigene vaccine containing a non-americ cytotoxic-T-lymphocyte epitope confers limited protection against Listeria monocytogenes infection. *Infect. Immun.* 64, 1685–1693 (1996).
14. Satchidanandam, V., Zavala, F. & Moss, B. Studies using a recombinant vaccinia virus expressing the circumsporozoite protein of Plasmodium berghei. *Mol. Biochem. Parasitol.* 48, 89–99 (1991).
15. Sedegah, M. *et al.* Active and passive immunization against Plasmodium yoelii sporozoites. *Bull. World Health Organ.* 68 Suppl., 1009–1014 (1990).
16. Stickl, H. & Hochstein-Mintzel, V. Intracutaneous smallpox vaccination with a weak pathogenic vaccinia virus ("MVA virus"). *Munch. Med/ Wochenschr.* 113, 1149–1153 (1971).
17. Mayr, A. *et al.* The smallpox vaccination strain MVA: marker, genetic structure, experience gained with the parenteral vaccination and behavior in organisms with a debilitated defence mechanism. *Zentralbl. Bakteriol. B.* 167, 375–390 (1978).
18. Sutter, G. & Moss, B. Nonreplicating vaccinia vector efficiently expresses recombinant genes. *Proc. Natl. Acad. Sci. USA* 89, 10847–10851 (1992).
19. Sutter, G. *et al.* A recombinant vector derived from the host range-restricted and highly attenuated MVA strain of vaccinia virus stimulates protective immunity in mice to influenza virus. *Vaccine* 12, 1032–1040 (1994).
20. Symons, J. A., Alcami, A. & Smith, G. L. Vaccinia virus encodes a soluble type I interferon receptor of novel structure and broad species specificity. *Cell* 81, 551–560 (1995).
21. Alcami, A. & Smith, G. L. A soluble receptor for interleukin-1β encoded by vaccinia virus: a novel mechanism of virus modulation of the host response to infection. *Cell* 71, 153–167 (1992).
22. Lockyer, M. J., Davies, C. S., Suhrbier, A. & Sinden, R.E. Nucleotide sequence of the Plasmodium berghei circumsporozoite protein gene from the ANKA clone 2.34L. *Nucleic Acid Res.* 18, 376 (1990).
23. Robson, K.J.H. *et al.* Cloning and expression of the thrombospondin related adhesive protein gene of Plasmodium berghei. *Mol. Biochem. Parasitol.* 84, 1–12 (1997).
24. Jaffe, R.I., Lowell, G.H. & Gordon, D.M. Differences in susceptibility among mouse strains to infection with Plasmodium berghei (ANKA clone) sporozoites and its relationship to protection by gamma-irradiated sporozoites. *Am. J. Trop. Med. Hyg.* 42, 309–313 (1990).
25. Seguin, M.C. *et al.* Induction of nitric oxide synthase protects against malaria in mice exposed to irradiated Plasmodium berghei infected mosquitoes: involvement of interferon gamma and CD8+ T cells. *J. Exp. Med.* 180, 353–358 (1994).
26. Renggli, J. *et al.* Elimination of P. berghei liver stages is independent of Fas (CD95/Apo-I) or perforin-mediated cytotoxicity. *Parasite Immunol.* 19, 145–148 (1997).
27. Weiss, W.R. *et al.* Cytotoxic T cells recognize a peptide from the circumsporozoite protein on malaria-infected hepatocytes. *J. Exp. Med.* 171, 763–773 (1990).
28. Li, S. *et al.* Priming with recombinant influenza virus followed by administration of recombinant vaccinia virus induces CD8+ T-cell-mediated protective immunity against malaria. *Proc. Natl. Acad. Sci. USA* 90, 5214–5218 (1993).
29. Moss, B. *et al.* Host range restricted, non-replicating vaccinia virus vectors as vaccine candidates. *Adv. Exp. Med. Biol.* 397, 7–13 (1996).
30. Lanar, D.E. *et al.* Attenuated vaccinia virus-circumsporozoite protein recombinants confer protection against rodent malaria. *Infect. Immun.* 64, 1666–1671 (1996).
31. Alcami, A., & Smith, G. L. Vaccinia, cowpox and camelpox viruses encode soulble interferon-γ receptors with a novel broad species specificity. *J. Virol.* 69, 4633–4639 (1995)
32. Plebanski, M., Elson, C. J. & Billington Dependency on interleukin-1 of primary human *in vitro* T cell responses to soluble antigens. *Eur. J. Immunol.* 22, 2353–2358 (1992).
33. Though, D.F., Borrow, P. & Sprent, J. Induction of bystander T cell proliferation by viruses and type I interferon *in vivo*. *Science* 272, 1947–1950 (1996).
34. Khusmith, S. *et al.* Protection against malaria by vaccination with sporozoite surface protein 2 plus CS protein. *Science* 252, 715–718 (1991).
35. Plebanski, M., Aidoo, M., Whittle, H.C. & Hill, A.V. S. Precursor frequency analysis of cytotoxic T lymphocytes to pre-erythrocytic antigens of Plasmodium falciparum in West Africa. *J. Immunol.* 158, 2849–2855 (1997).
36. Hanke, T., Szawlowski, P. & Randall, R. E. Contruction of solid matrix-antibody-antigen complexes containing SIV p27 using tag-specific monoclonal antibody and tag-linked antigen. *J. Gen. Virol.* 73, 653–660 (1992).
37. Mackett, M., Smith G.L. & Moss B. General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes. *J. Virol.* 49, 857–864 (1984).
38. Carroll, M.W., & Moss, B. E. coli beta-glucuronidase (GUS) as a marker for recombinant vaccinia viruses. *Biotechniques* 19, 352–356 (1995).
39. Bodmer, H.C., Pemberton, R. M., Rothbard, J. B. & Askonas, B. A. Enhanced recognition of a modified peptide antigen by cytotoxic T cells specific for influenza nucleoprotein. *Cell* 52, 253–258 (1988).
40. Gavin, M.A. *et al.* Alkali hydrolysis of recombinant proteins allows for the rapid identification of class I MHC-restricted CTL epitopes. *J. Immunol.* 151, 3971–3980 (1993).
41. Davis, H.L. *et al.* Direct gene transfer in skeletal muscle: plasmid DNA-based immunization against the hepatitis B virus surface antigen. *Vaccine* 12, 1503–1509 (1994).
42. Rodrigues, M. *et al.* Influenza and vaccinia viruses expressing malaria CD8+ T and B cell epitopes. Comparison of their immunogenicity and capacity to induce protective immunity. *J. Immunol.* 153, 4636–4648 (1994).
43. Brunner, K.T., Mauel, J., Cerottini, J. C. & Chapuis, B. Quantitative assay of the lytic action of immune lymphoid cells on 51-Cr-labelled allogeneic target cells in vitro; inhibition by isoantibody and by drugs. *Immunology* 14, 181–196 (1968).